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Epitope analysis of glycoprotein I of pseudorabies virus

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A panel of 11 monoclonal antibodies (MAbs) raised against pseudorabies virus (PRV) was used to map epitopes on the virus glycoprotein I (gI). We employed three approaches to map epitopes on gI. By a competition binding assay, six groups of MAbs were defined as reacting with distinct antigenic domains on gI. To identify regions along the gI polypeptide chain encompassing the domains recognized by these MAbs, DNA fragments derived from the gI-coding region were cloned into pEX expression plasmids. The antigenic reactivity of the fusion proteins expressed in *Escherichia coli* was analysed by immunoblotting. Five antigenic domains were mapped within the first 238 amino acids of gI: domains A, B and D were mapped between amino acids 52 and 123 and domains C and E

between amino acids 78 and 238. One MAb, representing domain F, did not react with the expressed fusion proteins. To assess the precise location and amino acid sequences of the epitopes, overlapping nonapeptides covering the amino acid sequence 52 to 238 were synthesized. The antibody-binding activity of these peptides was tested by an ELISA (Pepscan-method). Three antigenic domains were mapped: domain A was localized to amino acids 64 to 73 and 75 to 84, domain B to amino acids 52 to 67 and domain D to amino acids 68 to 82. Four MAbs representing antigenic domains C, E and F did not react in the Pepscan. Finally, sera from pigs infected experimentally with PRV reacted with the fusion protein of plasmid ps1 (amino acids 52 to 238).

Introduction

Aujeszky's disease is economically one of the most important diseases of today's pig industry. The disease is caused by pseudorabies virus (PRV), a member of the Alphaherpesvirinae. Pigs are vaccinated to reduce economic losses. However vaccination generally does not prevent infection and establishment of latency, and therefore will not lead to the eradication of PRV (Van Oirschot & Gielkens, 1984). Therefore tests to differentiate between infected and vaccinated pigs are crucial for the control of PRV in countries where vaccination is widely practised. For that purpose ELISA techniques have been developed that are based on detection of antibodies to glycoprotein I (gI) of PRV (Van Oirschot *et al.*, 1986, 1988; Eloit *et al.*, 1989). The gI of PRV is expressed by all wild-type virus strains tested so far (Van Oirschot, 1989), whereas some vaccine virus strains and genetically engineered deletion mutants lack gI (Hampl *et al.*, 1984; Mettenleiter *et al.*, 1985a; Gielkens *et al.*, 1989). Programmes to control Aujeszky's disease will be based on the use of either gI-negative, gIII-negative (Kit *et al.*, 1987) or gX-negative vaccines (Marchioli *et al.*, 1987), the identification of infected pigs by detection of antibodies to gI and the removal of these infected pigs from the herds. To investigate the gI-specific immune response in pigs, we characterized and localized epitopes

on gI by a competitive ELISA, by immunoscreening parts of the gI sequence expressed in a prokaryotic expression system and by the Pepscan method (Geysen *et al.*, 1984, 1985).

Methods

Monoclonal antibodies (MAbs). MAbs were produced against PRV strains NIA-3 and Phylaxia, and were purified from ascites fluid by ammonium sulphate precipitation and diluted in phosphate-buffered saline (PBS) to a final protein concentration of approximately 7 mg/ml. MAbs were conjugated to horseradish peroxidase (HRPO), essentially as described by Wilson & Nakane (1978).

Competitive ELISA. ELISA plates from Behringwerke, coated with PRV antigen (Enzygnost Aujeszky) were used. Serial dilutions of MAbs-HRPO conjugates in PBS containing 0.05% Tween 80 were made to determine the highest dilution of conjugate yielding an absorbance at 450 nm (A_{450}) of 1.5 to 2.0 in the absence of a second unlabelled antibody. Twice that concentration of conjugate was used in the competition ELISA. Each of the competing unconjugated MAbs was diluted 1:50, 1:100 and 1:1000, in each of the MAb-HRPO dilutions. The mixtures were transferred to the wells of the ELISA plate (100 µl/well). After incubation for 1 h at 37 °C the plates were washed with 0.05% Tween in deionized water and 100 µl of the substrate 3,3',5,5'-tetramethylbenzidine (1 mg/ml) was added. The reactions were stopped by adding 100 µl of 0.5 M-H₂SO₄ and the plates were read at 450 nm in a Titertek multiscan plate reader. The A_{450} value in wells without competing antibody was set at 100%. When a MAb dilution decreased the A_{450} value of the conjugated MAb by >50% it was considered to show competition.

Recombinant DNA techniques. Unless mentioned otherwise, the recombinant DNA techniques were done essentially as described by Maniatis *et al.* (1982) or Davis *et al.* (1986). Restriction enzymes and DNA-modifying enzymes were used as described by the manufacturer. Plasmid DNA was prepared using the alkaline lysis method (Maniatis *et al.*, 1982). DNA restriction fragments were isolated from agarose gels by electroelution (Bio Trap, Schleicher & Schüll).

Construction of recombinant plasmids expressing *gI*. The pEX plasmid expresses inserted genes as a *cro*- β -galactosidase fusion protein. Expression of this gene is under control of the λ *Pr* promoter and is induced by inactivation of the temperature-sensitive *cl* repressor at 42 °C (Stanley, 1983; Stanley & Luzio, 1984). We transformed *Escherichia coli* strain pop 2136 (constructed by Dr Rabaud, Institut Pasteur, Paris) by the CaCl_2 transformation procedure. Transformation occurred after a heat shock of 5 min at 34 °C and 2 min on ice. Plasmids linearized by a single digestion were dephosphorylated with calf intestinal phosphatase. The *AhaIII/NruI* fragment (2055 bp isolated from *BamHI*-7 fragment of PRV strain NIA-3; Quint *et al.*, 1987) with the entire *gI* coding sequence and fragments derived from the *gI*-coding region, were cloned in the correct reading frame into the *SmaI* site of pEX1, pEX2 or pEX3. Fragments were made blunt if necessary by treatment with the Klenow fragment of DNA polymerase I.

Isolation of expression products. Expression of the pEX fusion proteins was induced by incubation of a 1.5 ml exponentially growing culture (optical density at 600 nm of approximately 0.25) of cells at 42 °C for 90 min. The expression products were purified as follows: cells were spun down (5 min at 6000 g), resuspended in 100 μl 50 mM-Tris-HCl pH 8.0, 50 mM-EDTA, 15% (w/v) sucrose and treated with lysozyme (1 mg/ml) for 10 min. After addition of 140 μl of 0.2% (w/v) Triton X-100 in 10 mM-Tris-HCl pH 8, DNA was degraded by adding 24 μl 1 M-MgCl₂ and 1 μl DNase (10 mg/ml). Incubation was at 37 °C until the suspension was no longer viscous. After spinning down the insoluble expression proteins, we resuspended the pellet in 250 μl of PBS.

Immunoscreening of expression products. A 5 μl sample of resuspended proteins was dissolved in lysis buffer (10 mM-Tris-HCl pH 8.0, 1 mM-EDTA, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol; Laemmli, 1970). The proteins were fractionated by 7.5% SDS-PAGE, followed by Western blotting, using the LKB Multiphor II Nova Blot system in 40 mM-glycine, 50 mM-Tris, 0.04% SDS (w/v) and 20% methanol (v/v). The nitrocellulose sheets (BA-85, Schleicher & Schüll) were washed twice in PBS containing 0.5% (w/v) gelatin, 0.1% (w/v) Triton X-100 (PBS-GT) incubated for 1 h at room temperature with antibodies (MAb diluted 1:1000 in PBS-GT). The filters were washed three times for 5 min in PBS-GT and incubated with rabbit anti-mouse IgG-HRPO conjugate for 1 h at room temperature. The filters were washed three times for 5 min in PBS-GT and once for 5 min in PBS and incubated in substrate solution [3'-3'-diaminobenzidine (0.5 mg/ml) and 0.001% H₂O₂] for about 5 min.

Sera were from specified pathogen-free pigs infected intranasally with 10³ p.f.u. of the PRV strain Sterksel or NIA-3, or from a pig vaccinated twice with a *gI*-negative vaccine (Auskimune). Sera were collected 11 weeks post-infection (p.i.). Pig sera were tested in a dilution of 1:200.

Pepscan. Overlapping nonapeptides starting from amino acid 52 through to 238 of the *gI* protein were synthesized and tested as described (Geysen *et al.*, 1984, 1985). The amino acid sequence employed was based on the sequence of PRV strain Rice (Petrovskis *et al.*, 1986). All MAbs were tested at a 1:150 dilution in an ELISA as described by Geysen *et al.* (1984, 1985).

Results

Topographical analysis of the *gI* antigenic domains by a competitive ELISA

The *gI* specificity of the MAbs was determined by a radioimmunoprecipitation assay and SDS-PAGE (data not shown). In the competitive ELISA all MAbs inhibited the binding of the homologous MAb-HRPO by more than 50% (Fig. 1). Using a dilution of 1:1000 for the competing MAbs, six antigenic domains were distinguished: domain A, represented by MAbs 1, 3 and 5; domain B, represented by MAbs 4, 8 and 11; domain C, represented by MAbs 6 and 9; domain D, represented by MAb 7; domain E represented by MAb 2; domain F represented by MAb 10. MAb 9 competed for the binding of MAb 6-HRPO in a 1:1000 dilution of the competing MAb, whereas MAb 6 competed with conjugated MAb 9 in a 1:50 dilution only. Reciprocal competition between MAb 7 and MAbs 1, 3 and 5 was only obtained in a 1:50 dilution of the competing MAb. These results indicate that antigenic domains A and D are closely linked. Reciprocal competition was found

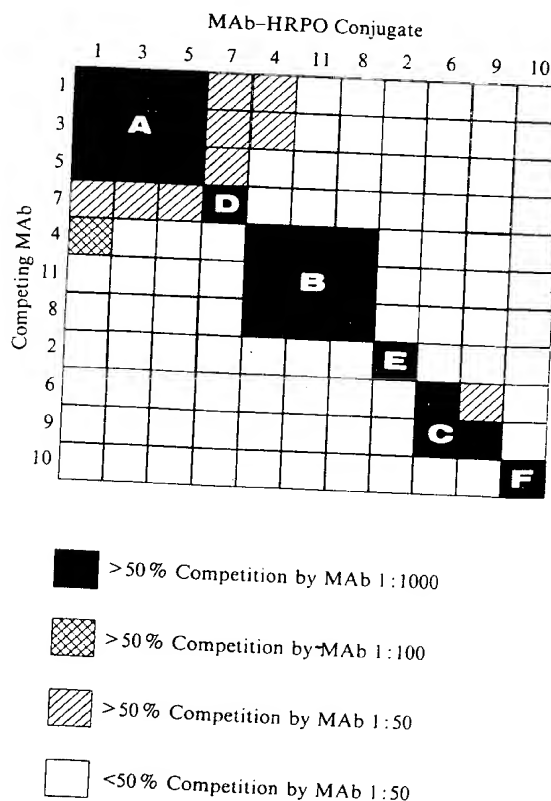


Fig. 1. Competitive ELISA. Each MAb was diluted 1:50, 1:100 and 1:1000 and allowed to compete for its epitope with each MAb-HRPO conjugate.

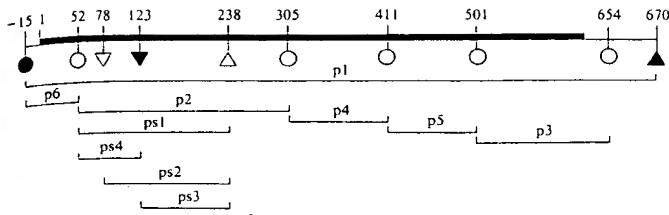


Fig. 2. Strategy for cloning the entire gI-coding sequence and fragments in pEX expression plasmids. Thick line indicates the gI open reading frame. Numbers indicate amino acid positions. Restriction sites: ●, *AhaIII*; ○, *NaeI*; ▼, *NcoI*; ▽, *MstII*; ▲, *NruI*; △, *SmaI*.

between MAb 4 and 1 at a 1:50 dilution, and MAb 3 and 4 competed in a non-reciprocal fashion at 1:50 dilution only.

Localization of antigenic domains by binding of gI-specific MAbs to gI-containing fusion proteins

An *AhaIII/NruI* fragment containing the entire gI-coding region was cloned in the proper reading frame into the pEX2 plasmid (plasmid p1 encoding 685 amino acids; Fig. 2). In the pEX expression system foreign gene fragments are inserted at the 3' end of the *cro-lacZ* gene, resulting in a hybrid protein of 115K (*cro*- β -galactosidase) plus the product of the foreign gene. The predicted size of the expression protein of plasmid p1 is 115K plus 80K (Mettenleiter *et al.*, 1985b). Analysis of the hybrid protein by SDS-PAGE revealed a protein band of about

Table 1. Reactivity of MAbs to pEX expression proteins of gI-coding fragments and in Pepscan*

MAb	pEX clones						Pepscan
	p1	p2	ps1	ps2	ps3	ps4	
1	+	+	+	-	-	+	+
2	+	+	+	+	-	-	-
3	+	+	+	-	-	+	+
4	+	+	+	-	-	+	+
5	+	+	+	-	-	+	+
6	+	+	+	+	-	-	-
7	+	+	+	-	-	+	+
8	+	+	+	-	-	+	+
9	+	+	+	+	-	-	-
10	-	-	-	-	-	-	-
11	+	+	+	-	-	+	+

* Reaction between MAb and expressed proteins and nonapeptide (in Pepscan) is shown by a plus (+) sign; no reaction, minus (-) sign.

190K (Fig. 3). In a Western blot 10 out of 11 MAbs tested recognized this protein (Table 1).

To map the antigenic domains on the protein the *AhaIII/NruI* fragment was digested with *NaeI* and, depending on the desired reading frame, the fragments were cloned into the *SmaI* site of pEX1, pEX2 or pEX3, resulting in the plasmids p2 (amino acids 52 to 305), p3 (amino acids 501 to 654), p4 (amino acids 305 to 411), p5 (amino acids 411 to 501) and p6 (amino acids -15 to 52). Only the fusion protein of plasmid p2 was recognized by the same MAbs which bound to the fusion protein of plasmid p1 (containing the entire gI coding sequence).

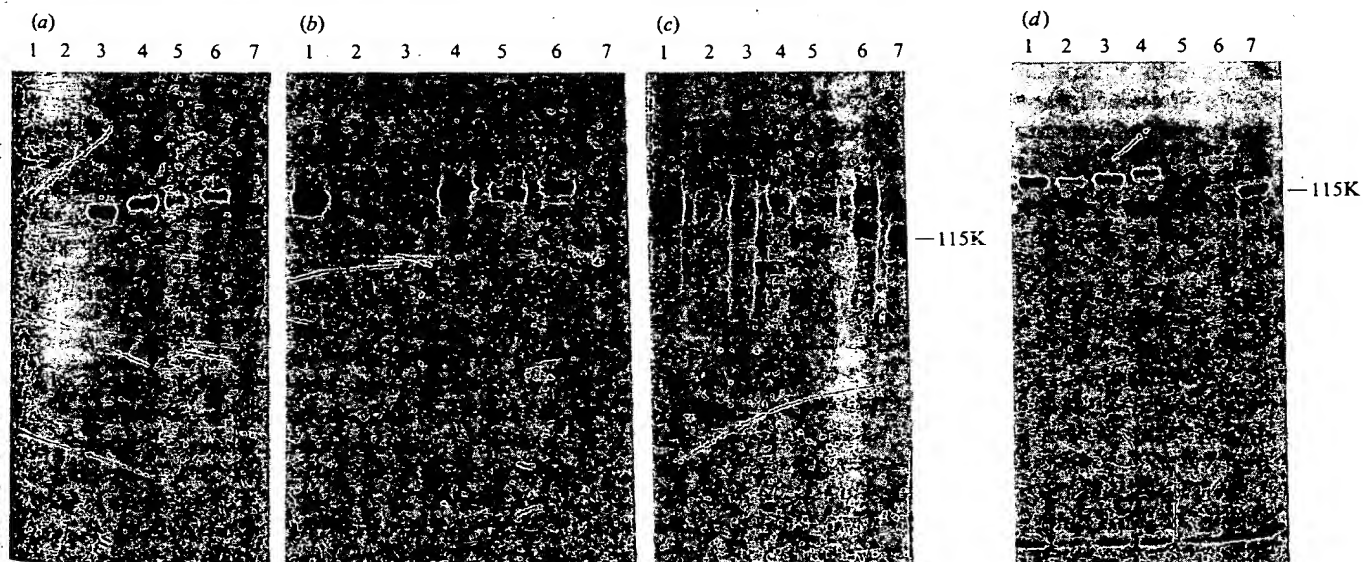


Fig. 3. Gel electrophoresis and Western blot of hybrid β -galactosidase proteins of different pEX clones. The M_r of hybrid protein from the pEX plasmid without an insert is 115K (lanes 7). (a) Western blot with MAb 2; (b) Western blot with MAb 8; (c) Western blot with murine anti- β -galactosidase MAb; (d) staining with Coomassie blue. Lanes 1, ps4; lanes 2, ps3; lanes 3, ps2; lanes 4, ps1; lanes 5, p2; lanes 6, p1.

These results indicate that the antigenic domains A, B, C, D and E were localized between amino acids 52 and 305. Subsequently fragments derived from the gI-coding DNA sequence of p2 were cloned into pEX (Fig. 2). In a Western blot the 10 MABs which recognized the fusion proteins of the plasmids p1 and p2, reacted also with the fusion proteins of the plasmid ps1 (amino acids 52 to 238). MABs 1, 3 and 5 (domain A), MABs 4, 8 and 11 (domain B) and MAB 7 (domain D) reacted with the fusion proteins of the plasmid ps4 (amino acids 52 to 123) and not with the fusion proteins of the plasmids ps2 (amino acids 78 to 238) and ps3 (amino acids 123 to 238). MAB 2 (domain E) and MABs 6 and 9 (domain C) reacted only with the fusion protein of plasmid ps2. These findings indicate that the antigenic domains A, B and D were localized between amino acid residues 52 to 123, probably between amino acid residues 52 to 78, and that the antigenic domains C and E were localized between amino acid residues 78 to 238.

The fusion protein of plasmid ps3 was not recognized by any of the gI-specific MABs tested (Table 1). The reactions of two MABs in the Western blot are shown in Fig. 3.

Localization of the epitopes by the Pepscan method

Overlapping nonapeptides (187) spanning the ps1 plasmid product (amino acids 52 to 238) were tested by Pepscan with the gI-specific MABs. MABs 1, 3 and 5 (antigenic domain A) reacted with peptides within the amino acid sequences 64 to 73 and 75 to 84. MABs 4, 8 and 11 (domain B) reacted with peptides within the amino acid sequence 52 to 67. MAB 7 (domain D) recognized peptides within the amino acid sequence 68 to 82 (Fig. 4b). The reaction patterns of MABs 1, 3 and 5 with all nonapeptides were similar as were the reaction patterns found for MABs 4, 8 and 11. The MABs 2, 6, 9 and 10 representing the antigenic domains C, E and F did not react with any of the synthesized peptides (Fig. 4a).

Reactivity of pig sera with the fusion protein of plasmid ps1

To investigate whether pigs produce antibodies against the antigenic domains determined by analysis with murine MABs, the fusion proteins of plasmid ps1 (amino acids 52 to 238) and the pEX plasmid without insertions were tested in a Western blot analysis with sera of PRV-infected pigs and pigs vaccinated with a gI-negative live vaccine. The reactions of the pig sera in the Western blot are shown in Fig. 5. All sera from infected pigs reacted with the fusion protein from plasmid ps1, but not with the expressed protein of the pEX plasmid without the

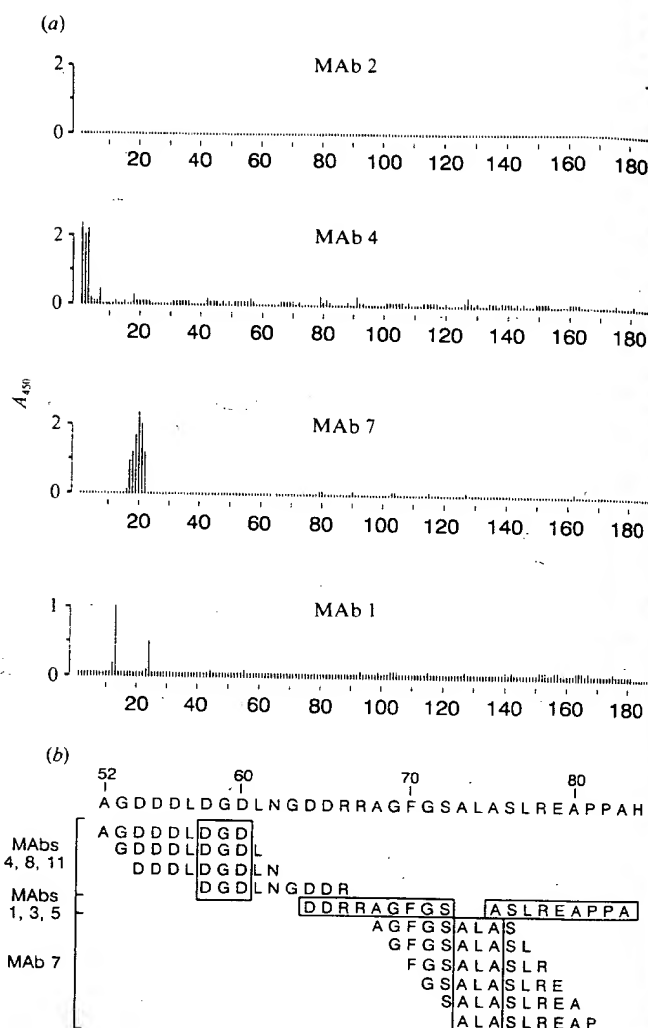


Fig. 4. Pepscan of PRV gI fragments against gI-specific MABs. Nonapeptides (187) starting from amino acid position 52 through to 238 were tested in an ELISA against 11 gI-specific MABs. (a) The reactivity of four MABs in the Pepscan is shown. The vertical lines indicate the absorbance value resulting from the reaction of the MABs with the nonapeptides. (b) The amino acid sequence of the region is represented by the single-letter code. The amino acid position is indicated by numbers. Amino acids present in all the overlapping nonapeptides of one epitope are boxed.

insert. The serum of the pig vaccinated twice with a gI-negative vaccine did not react with the fusion protein encoded by ps1.

Discussion

Tests for the detection of antibodies to gI are crucial for the eradication of Aujeszky's disease in the future (Van Oirschot *et al.*, 1986, 1988; Van Oirschot, 1989; Eloit *et*

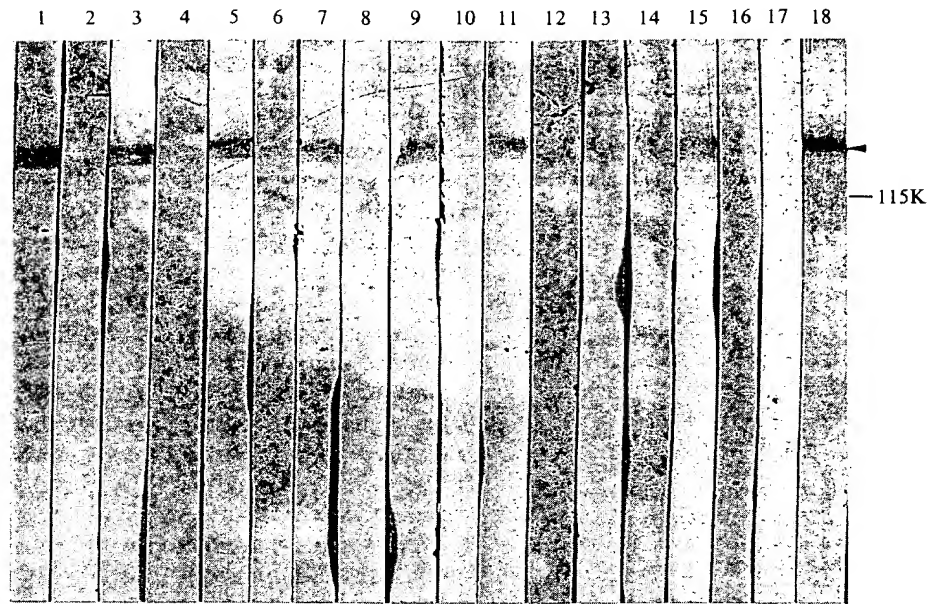


Fig. 5. Western blot analysis of pig sera with the fusion proteins of plasmid psI (amino acids 52 to 238) and of pEX plasmid without insertion. Lanes 1, 3, 5, 7, 9, 11, 13, 15, 17 and 18: Western blot of psI fusion protein. The fusion protein of the plasmid psI is indicated by an arrowhead. Lanes 2, 4, 6, 8, 10, 12, 14 and 16: Western blot of the expressed protein in the pEX plasmid without insertions. The M_r of the expressed protein is 115K. Lanes 1 to 8: incubation with sera collected 11 weeks p.i. from pigs infected with PRV strain Sterksel. Lanes 9 to 16: incubation with sera collected 11 weeks p.i. from pigs infected with PRV strain NIA-3. Lane 17: incubation with serum from a pig vaccinated with gI-negative vaccine (Auskimune). Lane 18: incubation with a MAb directed against gI.

al., 1989). For the development of an optimal detection system for gI-specific antibodies in pigs, knowledge about the epitopes on gI is necessary.

The competitive ELISA revealed the presence of six antigenic domains when a 1:1000 dilution of competing MAb was taken as a criterion. However, at a 1:50 dilution of competing MAbs the competitive ELISA revealed five domains. Antigenic domain D was then incorporated into the domain A; these two domains appear to be closely linked.

Indeed, the Pepscan analysis showed that these antigenic domains were overlapping (Fig. 4). The Pepscan showed also that domain B partly overlapped domain A. However the most important amino acids necessary for binding of the MAb to the peptides did not overlap (see Fig. 4b, boxed amino acids). In the competitive ELISA, the overlap resulted in only a weak competition of MAb 4 with MAbs 1 and 3.

Several MAbs did not compete reciprocally. Non-reciprocal competition may be explained by differences in affinity of the two MAbs, or by a conformational change induced by binding of one MAb that alters the binding of the other (Getzoff *et al.*, 1987). We also cannot exclude that the conjugating of a MAb to the enzyme alters its binding capacity.

By expression of subgenomic fragments in a prokaryotic expression system, λ gt11 or pEX for example

(Nunberg *et al.*, 1984; Mehra *et al.*, 1986; Luytjes *et al.*, 1989; Lenstra *et al.*, 1989), it is possible to localize antigenic domains on proteins. We have chosen to express relatively large subgenomic fragments in pEX to localize domains roughly and to use the Pepscan analysis to localize a number of epitopes precisely (Middeldorp & Meloen, 1988).

By the immunoscreening of fusion proteins in a Western blot it was possible to localize the antigenic domains A, B and D within the amino acid sequence 52 to 123 and domains E and C within amino acid sequence 78 to 238 at the N-terminal end of the gI protein. Localization of the epitopes at the N-terminal end of the protein is not unexpected because the C-terminal end of the gI protein contained the membrane anchor sequence (amino acids 429 to 453) and a hydrophilic region (amino acids 453 to 577) probably in contact with the interior of the virion (Petrovskis *et al.*, 1986).

None of the 11 MAbs, except perhaps MAb 10, were directed against epitopes in the region between amino acids 238 and 429. This suggests that this region may be less antigenic in the mouse, or it indicates an incompleteness of our panel of MAbs against gI, or it may be explained by a combination of these factors.

The Pepscan analysis showed that the antigenic domains B and D were continuous whereas domain A was discontinuous (Fig. 4). The three remaining domains

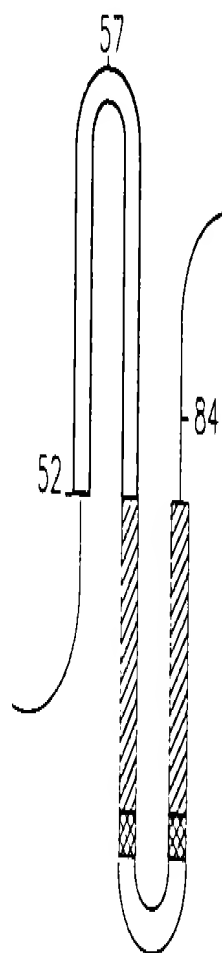


Fig. 6. A schematic representation of the localization of the MAb-binding sites. Hatched area (▨) indicates the binding site of MAbs 1, 3 & 5. Solid (■) indicates the binding site of MAbs 2 & 4.

less separated (discontinuous epitopes). This suggests a model (Fig. 6) for the secondary structure at this region of the protein. The turns in the protein structure were predicted by the method of Chou & Fasman (1978).

A noteworthy feature is that these epitopes were mapped by using murine MAbs. In theory the entire surface of a protein is antigenic; however the probability of obtaining antibodies to any given site varies greatly among different animals (Geysen *et al.*, 1987).

All sera from experimentally infected pigs contained antibodies which react with the fusion protein of the immunodominant region. None reacted with the protein expressed from the pEX plasmid. However when sera from pigs in the field were tested they reacted with the

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